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When chytrid doesn't kill: how it spread in túngara frogs and how females might avoid it

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When chytrid doesn't kill: how it spread in túngara frogs and how females might avoid it

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I dedicate this thesis to Nicolás.

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My dissertation aims to examine how pathogen-induced stress might affect reproductive behaviors such as sexual communication, mate choice, and reproductive success. To pursue this topic I studied the interaction between an emergent infectious disease, chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), and the tropical túngara frog as its host. The first goal of the dissertation was to understand the basic epidemiology of chytridiomycosis in this wide-spread tropical lowland anuran. From 2010 to 2015, I sampled annually for the presence of *B. dendrobatidis* in populations of túngara frog along an approximately 750 km transect, ranging from the mountains of western Panamá to inside the Darién Gap. Highland populations in western Panamá were already infected with *B. dendrobatidis* at the start of the study. In central Panamá, I collected the first positive samples in

2010, and by 2014, I detected *B. dendrobatidis* in samples from remote sites in eastern Panamá (Darién National Park) where *B. dendrobatidis* had not been documented before. I discuss the importance of studying *B. dendrobatidis* in lowland species, which may serve as potential reservoirs and agents of dispersal of *B. dendrobatidis* to highland species that are more susceptible to chytridiomycosis.

The second goal of my thesis was to understand how *B. dendrobatidis* might influence frog reproductive behavior. Some anuran species, including the túngara frog, seem to be tolerant to chytridiomycosis, but for others it is lethal. Tolerant species carry the pathogen, but do not exhibit symptoms of chytridiomycosis and their populations are not declining. Although chytridiomycosis might not be lethal for such tolerant species, it might nonetheless have other long-term effects. Such sub-lethal effects of chytridiomycosis have received little research attention. I examined how the potential pathogen-stress effects induced by *B. dendrobatidis* influence reproductive behavior such as sexual communication, mate choice, vigor, and reproductive success in the túngara frog. I tested the hypothesis that *B. dendrobatidis* influences the male mating call, and that females can use mating call cues to assess *B. dendrobatidis* infection. I performed female phonotaxis experiments to determine if males infection with *B. dendrobatidis* influences female mate choice, and I determined if there is a cost of the response to the infection in offspring number and development.

Overall, the research presented here improves our understanding of the

physiological and behavioral trade-offs confronted by a species during response to a pathogen and shows that *B. dendrobatidis* can have long-term population-level effects in tolerant species that are not severely affected by the disease. In addition to frogs and salamanders, emerging infectious diseases affect a number of other important lineages including honeybees, bats, birds, and humans. Study of the effects of non-lethal infections might therefore have more general application towards our understanding of the interactions between devastating pathogens and their wildlife hosts.

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Chapter 1

Introduction

We rarely think about diseases as a cause of extinction. There are very few examples of diseases responsible for massive mortalities that could lead to the complete extirpation of species (MacPhee & Greenwood, 2013). One case is the fungal pathogen *Batrachochytrium dendrobatidis*, which causes chytridiomycosis – an infectious disease in amphibians. *B. dendrobatidis* was isolated in 1999, from a poison-dart frog that died in captivity (Longcore et al., 1999). At that time, there was already mounting evidence that one of the causes of enigmatic declines of frog populations in North and Central America and Australia was a chytrid fungal infection on the skin of morbid frogs. Chytridiomycosis affects the skin of amphibians where it causes osmoregulatory irregularities which are though to be the cause of death (Voyles et al., 2009). This disease is believed to be responsible for the greatest vertebrate mass-mortality and extinctions in human history – the global demise of amphibians. Although different strains of *B. dendrobatidis* differ in pathogenicity (Bataille et al., 2013; Farrer et al., 2011; Flechas et al., 2013; Gabor et al., 2013), it is thought that amphibians' declines worldwide are caused by a global panzootic lineage spread across all continents (Fisher et al., 2009).

In the tropics, the most severe declines have been documented above 500 m, where temperatures are comparatively cooler and humid (Brem & Lips, 2008). During the 1990's, amphibian declines throughout the highlands of Central America were well documented (Lips, 1998, 1999; Pounds & Crump, 1994; Pounds et al., 1997). *B. dendrobatidis* was spreading from the west to the east in a wave-like pattern (Lips et al., 2008). High elevation sites in western and central Panamá were then intensively studied prior to and during the arrival of *B. dendrobatidis* (Lips, 1998, 1999; Lips et al., 2006; Woodhams et al., 2008). However, only two studies have investigated how *B. dendrobatidis* spread in the lowlands, where species were infected but declines or extinction were not observed (Rebollar et al., 2014; Woodhams et al., 2008). Species carrying the infection but resistant to varying degrees could be reservoirs of the pathogen, and potentially act as dispersers to more vulnerable populations in the highlands (Woodhams et al., 2008).

The túngara frog (*Physaelemus pustulosus*) is one of the most common and widely distributed species in the lowlands of Panamá. They inhabit the lowlands of Central America, ranging from Mexico to Colombia, and Venezuela, where they mate in permanent and ephemeral bodies of water in natural, rural and even urban areas. Túngara's geographic range parallels highland regions where *B. dendrobatidis* and amphibian declines have been extensively documented (Lips et al., 2008). Túngara frogs are infected with *B. dendrobatidis,* the pathogen can be detected in their skin using molecular assays, but they lack any clear signs of the disease and appear tolerant to it.

Túngara's broad geographic distribution, abundance, and tolerance to *B. dendrobatidis*, makes them a good model organism in which to study the dispersal of the pathogen as well as the important, but understudied, sub-lethal effects of the disease. My dissertation is focused on these topics.

The aim of the second chapter was to understand the basic epidemiology of chytrid infections in the widespread tropical lowland túngara frog. I describe the dynamics of the spread of *B. dendrobatidis* in populations along the lowlands of eastern Panamá from 2009 to 2014. After this six-year survey, I report that *B. dendrobatidis* had infected túngara frog populations throughout the lowlands of western Panamá. The resulting data on distribution and first occurrences of *B. dendrobatidis* during the survey from east to west Panamá also allowed me to characterize the rate of spread of *B. dendrobatidis* in a tolerant, lowland species, which could serve as a potential reservoir and disperser of the pathogen to more vulnerable species.

The effects of chytridiomycosis, as in the case of most diseases, are context-dependent, changing in response to the interaction between the environment, the identity of the host, and the pathogen strain. It is thought that amphibian declines are caused by a hypervirulent global panzootic lineage (Berger et al., 2016; Farrer et al., 2011; Gahl et al., 2012; Rosenblum et al., 2013) and certain species seem to be tolerant or resistant to the pathogen (Brem & Lips, 2008; Ellison et al., 2015). The complex life cycle of amphibians and the wide variety of life histories add to the complexity of this interaction (Stockwell et al., 2010). Few studies have demonstrated sub-lethal effects of

B. dendrobatidis in species that can tolerate the infection. These effects have consequences on individuals' development and performance at different life stages (An & Waldman, 2016; Gabor et al., 2013; Garner et al., 2009; Parris et al., 2006; Peterson et al., 2013; Roznik et al., 2015).

Studies on these sub-lethal effects have shown behavioral changes associated with the infection by *B. dendrobatidis* (Parris et al., 2006; Gabor et al., 2013; Garner et al., 2009; An & Waldman, 2016). Infected tadpoles of *Rana pipiens* exhibit decreased foraging activity when compared to uninfected ones. This, in turn, results in higher survivorship of infected tadpoles in the presence of a predator (Parris et al., 2006). Physiological changes due to infection with *B. dendrobatidis* have also been studied in tadpoles. Chytridiomycosis elevates the concentration of corticosterone in infected tadpoles of two species of *Alytes* (Gabor et al., 2013) and in adults of *Litoria caerulea* (Peterson et al., 2013). Moreover, Garner et al. (2009) have shown that the life history stage and the body size after metamorphosis are critical for the survivorship of *Bufo bufo* in the presence of the pathogen. These results suggest that there is a cost to the infection with *B. dendrobatidis* with direct consequences for fitness, possibly due to pathogen induced stress, even when *B. dendrobatidis* does not kill the host. In adults, changes in the characteristics of male's mating call in *Hyla japonica* have been associated with the infection (An & Waldman, 2016). In *L. rhaecola* the characteristics of the males' mating calls did not differ between infected and uninfected males, but the infected males were more likely to call if they were in good body condition (Roznik et al., 2015). Overall, although data

are still scarce, these studies lend support to the hypothesis that infection with *B. dendrobatidis* has non-lethal effects that can be reflected in various aspects of behavior and are related to the energetic state and vigor of individuals.

Calling behavior in frogs is metabolically very costly (Gerhardt & Huber, 2002). In most frog species females assess the male's acoustic sexual signal and decide with whom to mate. Túngara frogs' calling behavior and female choice are well documented (Ryan, 1985). This species is one of the best-studied tropical frogs in the world, especially in terms of behavior, physiology, neurophysiology, and the mechanisms underlying sexual selection (Lynch et al., 2006; Ryan, 1985, 2010, 2011; Ryan & Rand, 1990). Male túngara frogs produce a whine, which females use to recognize members of the same species, and then add one or more chucks, which further increase the caller's attractiveness to females (Rand & Ryan, 1981). As a well-established model organism in behavioral studies, that appears tolerant to *B. dendrobatidis*, the túngara frog emerges as an excellent system to investigate the interaction between *B. dendrobatidis* and sexual behavior of species that are not under immediate threat.

In the third chapter, I examined how the potential pathogen-stress effects induced by infection with *B. dendrobatidis* interact with reproductive behaviors such as mate choice. Specifically, I tested whether female túngara frogs are able to discriminate between infected and uninfected males by attending to the male's acoustic signal. My hypothesis was that the cost of the response to the infection with *B. dendrobatidis* incurs a trade-off in the ener-

getic state of túngara males, affecting the production of males' sexual mating signal and thus female preference.

This is the first study to test the ultimate effects of changes in calling behavior and mate choice as a consequence of the infection with *B. dendrobatidis.* Females' ability to discriminate between males based on the infection status of a male could spare the females from infection with the pathogen, and could further reduce the rick of transmitting the infection the offspring. The infective life stage of the *B. dendrobatidis* is a waterborne flagellated zoospore released from the skin surface upon completion of the life cycle. A couple of tungara frogs in amplexus will lay and fertilize eggs in standing bodies of water and remain in proximity to the foam nest for about one hour. This period of close proximity in standing water can increase the chances of infection of tadpoles potentially affecting their growth and development. In the fourth chapter, I test whether pathogen stress effects, due to infection with *B. dendrobatidis* in adults, are carried on to the next generation affecting both the adults' reproductive output and the body size of tadpoles.

Chapter 2

Spread of amphibian chytrid fungus across lowland populations of túngara frogs in Panamá

2.1 Abstract

Chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), is an emergent infectious disease partially responsible for worldwide amphibian population declines. The spread of *B. dendrobatidis* along highland habitats [$>$ 500 meters above sea level (m a.s.l.)] of Costa Rica and Panamá is well documented and has been linked to amphibian population collapses. In contrast, data are scarce on the prevalence and dispersal of *B. dendrobatidis* in lowland habitats where amphibians may be infected but asymptomatic. Here we describe the spread (2009 to 2014) of *B. dendrobatidis* across lowland habitats east of the Panamá Canal ($<$ 500 m a.s.l.) with a focus on the túngara frog (*Physalaemus* [*Engystomops*] *pustulosus*), one of the most common and abundant frog species in this region. Highland populations in western Panamá were already infected with *B. dendrobatidis* at the start of the study, which was consistent with previous studies indicating that *B. dendrobatidis* is enzootic in this region. In central Panamá, We collected the first positive samples in 2010, and by 2014, we detected *B. dendrobatidis* from remote sites in eastern Panamá (Darién National Park). We discuss the importance of studying *B.*

dendrobatidis in lowland species, which may serve as potential reservoirs and agents of dispersal of *B. dendrobatidis* to more susceptible highland species.[1]

2.2 Introduction

Wildlife extinctions are not typically attributed to infectious diseases, yet there are a few examples showing pathogens as the direct cause of local extinctions (MacPhee & Greenwood, 2013; McCallum, 2012; Smith et al., 2006). Some of them have been linked to chytridiomycosis, an emerging infectious disease (Fisher et al., 2012; Pounds & Crump, 1994). In amphibians, chytridiomycosis results from a skin infection caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*B. dendrobatidis*). In the tropics, the most severe declines have been documented in comparatively cooler and humid areas above 500 m (Brem & Lips, 2008), most likely because lower temperatures ($17-25^{o}$C) are optimal for *B. dendrobatidis* growth and water facilitates the propagation and dispersal of the aquatic, flagellated *B. dendrobatidis* zoospores (Piotrowski et al., 2004). Although the rapid spread of *B. dendrobatidis* into apparently *B. dendrobatidis*-free areas throughout the world is well-documented (Farrer et al., 2011; Fisher et al., 2009; James et al., 2009; Lips et al., 2008; Velo-Antón et al., 2012), the process by which *B. dendrobatidis* spreads is still not clearly under-

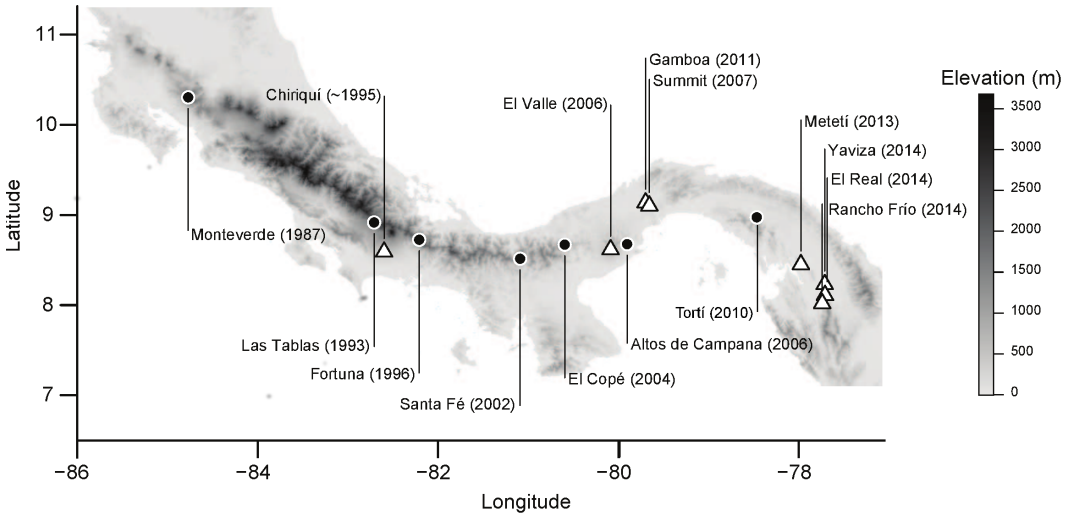
[1]This chapter was published as Rodríguez-Brenes S., D. Rodriguez, R. Ibáñez, M. J. Ryan, 2016. PLoSONE. Author contributions: Conceived and designed the experiments: SRB MJR. Performed the experiments: SRB MJR RI DR. Analyzed the data: SRB DR RI. Contributed reagents,materials,analysis tools: SRB MJR DR RI. Wrote the paper: SRB MJR DR RI.

stood (Phillips & Puschendorf, 2013).

B. dendrobatidis has rapidly spread throughout the highlands of Central America. In the late 1980s, the disappearance of golden toads (*Incilius periglenes*) and declines of other anuran populations in the protected cloud forest of Monteverde, Costa Rica (Fig. 2.1), were the first alerts to what later became a predictable pattern of declines spreading towards Panamá (Pounds & Crump, 1994; Pounds et al., 1997). Shortly thereafter, between 1993 and 1997 additional cases of amphibian population declines or extinctions were reported in the highlands close to the border with Panamá at Las Tablas, Costa Rica, and Fortuna, Panamá (Fig. 2.1)(Lips, 1999, 1998). The sites experiencing population declines were located at altitudes above 500 m, and the fastest declining species were stream dwellers with aquatic tadpoles (Brem & Lips, 2008). *B. dendrobatidis* was spreading west to east in a wave like pattern (Lips et al., 2008). High elevation sites in western and central Panamá were then intensively studied prior to and during the arrival of *B. dendrobatidis*, these studies documented amphibian population declines after the arrival of *B. dendrobatidis* in this region (Fig. 2.1)(Lips, 1998, 1999; Lips et al., 2006; Woodhams et al., 2008).

Only two studies have investigated *B. dendrobatidis* infection spread in eastern Panamá. Rebollar et al. (2014) sampled populations along the lowlands of east Panamá, and Woodhams et al. (2008) sampled sites in central Panamá. These studies did not detect a clear pattern of wave-like spread from west to east as observed in the highlands of Panamá. It is also possible that

Figure 2.1: Sites where *B. dendrobatidis* has been detected in the past (filled circles), and sites where we sampled túngara frog (*Physalaemus* [*Engystomops*] *pustulosus*) populations (open triangles). Year in parenthesis corresponds to year of decline or the year that *B. dendrobatidis* was first detected.



another wave of *B. dendrobatidis* from South America crossed into Panamá (Lips et al., 2008). Currently, the dynamics of *B. dendrobatidis* spread in the lowlands of Panamá, where environmental conditions are not ideal for *B. dendrobatidis*, are still unclear and require additional investigation.

Evaluating the spread of a pathogen in an abundant and well-characterized host can help to predict spread dynamics while controlling for host phylogenetic diversity. The túngara frog (*Physalaemus* [*Engystomops*] *pustulosus*) is a common species occupying lowland habitats ranging from México to Colombia, Venezuela, and a small portion of the Guyana Shield. Its range parallels many highland regions where *B. dendrobatidis* and amphibian declines have been

extensively documented (Farrer et al., 2011; Fisher et al., 2009; James et al., 2009; Lips et al., 2008; Phillips & Puschendorf, 2013; Velo-Antón et al., 2012). The adults and tadpoles of this species use both permanent and ephemeral bodies of water in habitats ranging from urban areas to pristine forests. Thus, the túngara frog is an ideal species in which to characterize the spread of *B. dendrobatidis* through populations of a lowland host. Here, we (i) report the *B. dendrobatidis* infection of túngara frogs at two sites west of the Panamá Canal where *B. dendrobatidis* is enzootic, and (ii) present data documenting the spread of *B. dendrobatidis* in the lowlands of central and east Panamá from 2009 to 2014.

2.3 Methods

We sampled túngara frog populations in Panamá from 2009 to 2014, each year during the rainy season (June to November), which falls within their reproductive season. In most cases, populations were sampled during two reproductive seasons. Lowland sites in east and central Panamá ranged from 12 to 67 m in altitude (Fig. 2.1). We also sampled highland populations at El Valle (elevation 600 m; Fig. 2.1) in central Panamá, and at Chiriquí near the town of Cuesta de Piedra (elevation 460-967 m; Fig. 2.1) in western Panamá. This is the highest altitude at which túngara populations have been documented in Panamá. Chiriquí was also our westernmost site and was located 30 km east of sites where other amphibian species experienced declines in 1993 (Lips,

1998).

In central Panamá, we sampled two lowland sites east of the Panamá Canal, Gamboa and Summit, ranging from 46 to 98 m in elevation (Fig. 2.1). These two lowland sites are located on the eastern side of the Panamá Canal but are separated by the Chagres River. Gamboa is a small town north of the river that is surrounded by rainforest. Here we included Pipeline Road, which intersects a protected rainforest in the Soberanía National Park. Summit is located south of the river and includes portions of the Soberanía National Park. We sampled along the main roads, on trails, and dirt roads within the National Park.

In east Panamá, we sampled at four sites. Metetí and Yaviza are located along and at the very end of the Inter-American Highway, respectively (Fig. 2.1). Here, we sampled disturbed, deforested habitats, in puddles on dirt roads and cattle ranches. Further east, along the Tuira River, we sampled at El Real (Fig. 2.1), a small town surrounded by less disturbed habitat. Farthest east, in the Darién National Park we sampled around the Rancho Frío field station (Fig. 2.1) at an average of 50 m of elevation. This site is predominantly primary rainforest with a tall canopy, thick lianas and an understory dominated by palms.

2.3.1 Time scale of surveys

We first sampled El Valle in 2009 and then Chiriquí in 2010, where *B. dendrobatidis* is thought to have arrived in the mid-1990s according to previous

estimations (Phillips & Puschendorf, 2013). In 2010, we also sampled Gamboa and Summit in central Panamá. Woodhams et al. (2008) reported *B. dendrobatidis* in Summit in 2007. We then sampled Metetí and Yaviza in eastern Panamá during 2011, where we expected the front of the *B. dendrobatidis* wave to be just arriving. In 2013, we sampled at all locations previously surveyed. In 2014 we sampled in Gamboa, Yaviza and finally the Darién National Park, which was the easternmost site and where we expected *P. pustulosus* populations to be *B. dendrobatidis* naive.

2.3.2 *B. dendrobatidis* sampling

We toe-clipped individuals to avoid recapture. To avoid cross contamination, we captured adults by hand using a new pair of nitrile gloves for each individual and kept them isolated in a plastic bag until processing. We swabbed the ventral area using a sterile cotton tip dry swab (Medical Wire & Equipment, model MW113 and MW110) following established procedures (Hyatt et al., 2007). Swabs were stored in 90% ethanol or they were kept frozen until DNA extraction, thus we expect no effects of sample storage on estimates of prevalence or infection intensity (Sluys et al., 2008). To avoid potential cross-contamination between sites, we bleached our rubber boots and vehicle tires and then rinsed them with tap water before leaving the collection site.

2.3.3 Real time quantitative PCR

To determine the prevalence of *B. dendrobatidis* in each population and the intensity of infection for individual frogs, we processed the swabs using quantitative PCR (qPCR) following the protocol developed by Boyle et al. (2004) and modified by Kriger et al. (2006). For samples collected from 2009 to 2012, we used a Roche LightCycler 480 system with a high confidence setting to detect positive samples. For samples collected during 2009-2010, we did not quantify the number of zoospores due to lack of *B. dendrobatidis* standards. For samples collected during 2013 and 2014, we used TaqMan®Fast Advanced Master Mix (Applied Biosystems) and a StepOnePlus^{TM} system. We used a dilution series of genomic DNA from strain JEL423 as our standard reference for the estimations of infection intensity in samples collected from 2011 to 2014. We calculated prevalence by using the ratio of positive samples to total samples per population and calculated 95% binomial confidence intervals. For each population, we calculated average infection intensity using the average number of zoospore equivalents (z.e.) inferred from qPCR among positive individuals.

To calculate the rate of *B. dendrobatidis* spread across sites east of the Canal, we used the distance between sites and the year we first detected infected frogs. We sampled frogs from June to November. If *B. dendrobatidis* arrived after sampling was completed for a given year, then we would have detected it the following year. We could not calculate the rate of spread in western Panamá because *B. dendrobatidis* was already present at the begin-

ning of our sampling period. Thus, we approximated when túngara frogs were first infected based on historical reports for sites near Chiriquí (Lips et al., 2008; Phillips & Puschendorf, 2013; Woodhams et al., 2008).

2.3.4 Ethical approval

All applicable institutional and/or national guidelines for the care and use of animals were followed. The protocol was approved by the Institutional Animal Care and Use Committee of the Smithsonian Tropical Research Institute (protocol number: 2011-0825-2014-02), and by the Autoridad Nacional del Ambiente (permit numbers: SE/A-81-09, SE/A-73-10, SE/A-48-10, SC/A-28-11, SE/A-83-11, SE/A-42-11, SE/A-30-12, SE/A-47-13, SC/A-9-14).

2.4 Results

In total, we sampled 1695 *P. pustulosus* adults across Panamá from 2009 to 2014 (Fig. 2.1). Sample size, prevalence, and 95% binomial confidence intervals are shown in Table 2.1. We considered sites with sample sizes greater than 60 individuals and no positive samples as *B. dendrobatidis*-negative (Skerratt et al., 2008). The site Chiriquí, in western Panamá, was positive for *B. dendrobatidis* in 2010 and 2013. In central Panamá, El Valle was positive for *B. dendrobatidis* in 2009. In 2010, Gamboa samples were negative for *B. dendrobatidis*, but Summit, which is only 8 km to the south, was positive. *B. dendrobatidis* reached Gamboa in 2011, and in following years we detected *B. dendrobatidis* positive samples in both Summit and Gamboa. By 2014,

prevalence had reached 26% in Gamboa (Table 2.1). Populations in Metetí were *B. dendrobatidis* naive in 2011 but positive in 2013. Farther east, Yaviza was naive for *B. dendrobatidis* in 2013, but by 2014 prevalence reached approximately 6%. By 2014, *B. dendrobatidis* was also present in *P. pustulosus* populations from El Real and Rancho Frío, Darién National Park (Table 2.1).

2.4.1 Rates of spread of *B. dendrobatidis* in túngara frogs

If *B. dendrobatidis* spread eastward from Summit in central Panamá to Darién in eastern Panamá, then the front moved at an average rate of 54 km/year among these lowland túngara frog populations. The rates of spread, however, vary substantially. Specifically, our data suggest that it took approximately one year for *B. dendrobatidis* to move the 8 km distance between Summit and Gamboa, which are separated by the Chagres River. In contrast, the rate of *B. dendrobatidis* spread from Summit to Metetí was 65 km/year, and 42 km/year from Metetí to Yaviza. All data are available from the DRYAD Digital Repository (doi:10.5061/dryad.6bp92).

Table 2.1: *B. dendrobatidis* (Bd) prevalence and infection intensity per site and year in túngara frog (*Physalaemus* [*Engystomops*] *pustulosus*) populations sampled in this study. Sites are arranged west to east by longitude.

Site	Year	N[a]	Positive[b]	Prevalence % (95% CI[d])	Average Intensity[c] (± St.Dev)
Chiriquí	2010	41	11	27 (14-43)	data not available
	2013	38	16	42 (26-60)	3570 (± 12454)
El Valle	2009	5	3	60 (15-95)	data not available
Gamboa	2010	321	0	0 (0-0.5)	0
	2011	111	7	6 (3-13)	86 (± 117)
	2012	205	26	13 (8-18)	1617 (± 5485)
	2013	166	35	21 (15-29)	536 (± 2061)
	2014	84	22	26 (17-37)	209 (± 833)
Summit	2010	12	2	17 (2-48)	data not available
	2011	108	2	2 (0-6)	10 (± 7)
	2013	120	17	14 (9-22)	89 (± 193)
Metetí	2011	91	0	0 (0-4)	0
	2013	94	2	2 (0-8)	7 (± 3)
Yaviza	2013	68	0	0 (0-0.5)	0
	2014	41	3	7 (2-20)	6 (± 1)
El Real	2014	40	2	5 (1-17)	6 (± 1)
Rancho Frío	2014	150	11	7 (4-13)	48 (± 101)

[a]Total number of sampled individuals
[b]Number of individuals detected positive for *B. dendrobatidis*
[c]Average of number of zoospore equivalents in infected frogs per population
[d]95% binomial distribution confidence intervals

2.5 Discussion

Data for the spread of *B. dendrobatidis* in the lowlands of Middle America are scarce. Here we estimated the rate of spread of *B. dendrobatidis* in túngara frogs based on the first detection of *B. dendrobatidis*, and we assumed that *B.*

dendrobatidis spread in a wave-like fashion (Lips et al., 2008). In the lowlands of Panamá, *B. dendrobatidis* spread at a similar rate among túngara frogs (54 km/year) when compared to other amphibian species tested in this area (30-174 km/year) (Woodhams et al., 2008). There was one exception. The rate of spread from Summit across the Chagres River to Gamboa was slower than the average (8 km/year).

In western Panamá, we sampled in Chiriquí, at elevations where amphibian population declines have been severe in the past (Lips, 1999). Since *B. dendrobatidis* is now enzootic in this area (Woodhams et al., 2008) and at El Valle in central Panamá, it is not surprising that the túngara frog populations from Chiriquí and El Valle were positive in 2010, and 2009, respectively. We also found the highest average loads of *B. dendrobatidis* in Chiriquí (3570 zoospore equivalents, z.e., Table 2.1). We could not estimate the rate of *B. dendrobatidis* dispersal among túngara frog populations between Chiriquí and El Valle as they were already infected at the time we sampled. In 2007, Woodhams et al. (2008) reported *B. dendrobatidis* positive individuals (30% prevalence) among three species at Soberanía National Park, including the Summit area where we sampled in 2010, and they suggested that *B. dendrobatidis* was already enzootic during their study. In the same year, Gamboa, was still naive and we most likely sampled before *B. dendrobatidis* arrived. In 2011, we detected *B. dendrobatidis* at Gamboa for the first time, one year earlier than previously reported for this area (Rebollar et al., 2014).

We cannot discern whether the source of infection of the Gamboa pop-

ulations was from Summit túngara frogs or from other species of frogs in Gamboa. Regardless, there is a relatively large time lag between when *B. dendrobatidis* was detected in túngara frogs from Gamboa compared to when *B. dendrobatidis* was first detected in túngara frogs from Summit. The mechanism by which *B. dendrobatidis* spreads is unknown, but it has been suggested that *B. dendrobatidis* could survive and could be carried in mud, and thus easily dispersed by humans (Johnson & Speare, 2005). In túngara frogs, *B. dendrobatidis* did not spread as fast as expected from Summit to Gamboa, which are only 8 km apart and connected by a well-traveled road and bridge over the Chagres River.

Certain geographic features, like rivers, could impede the spread of *B. dendrobatidis*. The Chagres River, which is about 100 m wide and has water all year long, separates Gamboa and Summit. Genetic studies demonstrate that the Chagres River is a geographical barrier for gene flow between túngara frog populations (Lampert et al., 2003), thus it is possible that limited migration between these populations could slow the spread of *B. dendrobatidis* whether túngara frogs contracted *B. dendrobatidis* from conspecifics or heterospecifics. *B. dendrobatidis* does seem to have spread rapidly in the lowlands towards eastern Panamá. An alternative explanation for the spread of *B. dendrobatidis* from east to west throughout all of Panamá is that populations in Darién, and elsewhere in far eastern Panamá, were infected by a wave coming from the south (Lips et al., 2008). These two scenarios should be tested through a phylogenetic analysis of *B. dendrobatidis* throughout the túngara frog's range

in Central and South America. As such data are currently unavailable, it is difficult to determine the recent origin of *B. dendrobatidis* in eastern Panamá; therefore, our estimates of rate of spread should be viewed with this potential caveat in mind.

Woodhams et al. (2008) conservatively estimated that *B. dendrobatidis* spread east and would have reached Tortí in September of 2012, but Küng et al. (2014) reported two positives out of 93 samples of other species of frogs at this site in 2010. There are no other published reports on the presence of *B. dendrobatidis* among amphibian species from this specific area. In 2011, we sampled in Metetí, approximately 70 km southeast of Tortí, and found this site to be *B. dendrobatidis* naive. Rebollar et al. (2014) recorded *B. dendrobatidis* positive samples from Nuevo Vigía in 2012, just 26 km east of Metetí, where we detected low *B. dendrobatidis* prevalence in 2013, thus supporting a wave-like *B. dendrobatidis* spread from west to east in túngara frogs.

Species that carry *B. dendrobatidis* asymptomatically and share the habitat with more vulnerable species can potentially function as *B. dendrobatidis* spreaders. Túngara frog population declines have not been reported and were not evident during our study. If the prevalence and dispersal of *B. dendrobatidis* is density dependent, then the spread of *B. dendrobatidis* along the lowlands might be enhanced by abundant and apparently resistant species serving as reservoirs. Túngara frogs are known to disperse between breeding sites at distances up to 200 m (Marsh et al., 1999). They also share the habitat with a wide variety of species; thus, they could contribute to the rapid disper-

sal of *B. dendrobatidis* if they are effective carriers. Moreover, if most species in the lowlands are less susceptible to *B. dendrobatidis* or *B. dendrobatidis* is less virulent (Piotrowski et al., 2004), then the high diversity and abundance of hosts could further facilitate the dispersal of *B. dendrobatidis*.

Chytridiomycosis is linked to some of the most severe population declines and extinctions of wildlife yet recorded. While substantial efforts have been aimed at understanding the spread and pathogenicity in the more vulnerable highland frog species, we know relatively little about the dynamics of *B. dendrobatidis* in tropical lowland regions of the world. Lowland species, however, could be reservoirs and dispersal agents between areas where amphibian species are more vulnerable to *B. dendrobatidis* infection. As highlighted by our results, even though lowland regions are typically characterized by less favorable climatic conditions for *B. dendrobatidis* (Piotrowski et al., 2004), by harboring asymptomatic *B. dendrobatidis* infections, lowland amphibian populations could potentially play an important role in the spread of *B. dendrobatidis* across tropical regions.

2.6 Acknowledgements

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Chapter 3

Catastrophic emergent disease avoided by female mating preferences

3.1 Abstract

Amphibian species worldwide are disappearing in the most devastating mass extinction in human history and one of the six most profound extinctions in the history of life. One of the main causes of amphibian extinctions is the lethal disease chytridiomycosis, caused by a fungus. Although some species are somewhat tolerant of the disease, the non-lethal effects and their short or long term consequences are poorly understood. In these species there is the potential for behavioral responses to mitigate the spread of the fungus. Here we show that in túngara frogs infection status influences the males' mating calls, and we demonstrate that these infection-induced changes in the quality of males' advertisement calls ultimately reduce the calls' attractiveness to females making females less likely to respond to and mate with infected males. The broader implications of our results indicate that such recruitment of behavioral responses might potentially ameliorate some of the effects of this sixth mass extinction.[1]

[1]This chapter is in review for publication as: Sofía Rodriguez-Brenes, Sylvia Garza and Michael Ryan. Catastrophic emergent disease avoided by female mating preferences. Author

3.2 Introduction

Amphibian species are experiencing the largest mass extinction in human history and one of the six most profound extinctions in the history of life. An emergent disease, chytridiomycosis, has had devastating effects on amphibian populations across the world and is one of the main contributors to this catastrophic loss of biodiversity (Collins & Crump, 2009; Kolbert, 2014; Wake & Vredenburg, 2008; Ceballos et al., 2015). Chytridiomycosis, caused by the chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), is well known for its lethal effects. *B. dendrobatidis* disrupts osmoregulatory functions of amphibian skin ultimately causing cardiac arrest (Voyles et al., 2009) and it also inhibits the host's immune system (Fites et al., 2013). Amphibian hosts vary in their defense mechanisms to fight the infection from *B. dendrobatidis*; their skin microbiota may function as a first barrier against the fungi (Harris et al., 2009), and immune responses specific to *B. dendrobatidis* have also evolved in some amphibian populations (Ellison et al., 2015; Savage & Zamudio, 2011; Bataille et al., 2015)). In nature, some species are infected with *B. dendrobatidis* but are thriving and do not show any of the lethal symptoms of the disease, but some behavioral effects have been observed. In two species of frogs, tadpoles' foraging behavior decreased when infected with *B. dendrobatidis* (Venesky et al., 2009). In an Asian frog, changes in the characteristics of male's mating call have been associated with the infection (An & Waldman,

contributions: SRB and MR concieved the study and designed experiments. SRB and SG performed experiments. SRB performed statistical analyses. SRB and MJ wrote the paper.

2016) but the fitness consequences of these non-lethal effects are unknown.

Amphibians can contract *B. dendrobatidis* through contact with fungal spores in the environment and from contact with other infected individuals (Rachowicz & Vredenburg, 2004). *B. dendrobatidis* can be transmitted, for example, in both directions between tadpoles and adults and between conspecifics and heterospecifics (Fernández-Beaskoetxea et al., 2016). Given that *B. dendrobatidis* infects the skin, adult-adult contact is also assumed to mediate direct transmission of the disease. An especially dangerous venue for *B. dendrobatidis* transmission should occur during sex. In most frog species females assess the male's acoustic sexual signal and decide with whom to mate. Male túngara frogs produce a whine, which females use to recognize conspecifics, and then add one or more chucks, which further increases their attractiveness to females (Rand & Ryan, 1981). Mate choices are usually followed by a 'love hug' or amplexus which often lasts for hours allowing sufficient time for the transmission of the pathogen. Frog calls in general, and túngara calls in particular, are energetically very costly (Ryan, 1985). Thus a male's allocation of resources and energy to the immune system in order to cope with an infection could influence the amount of energy devoted to calling. This has been shown in other amphibians: in the treefrog, *Hypsiboas prasinus*, the presence of helminth parasites decreased the calling rate of males (Madelaire et al., 2013) and infection with *B. dendrobatidis*, in the frog *Litoria rheocola,* decreases the probability of calling in males with poor body condition (Roznik et al., 2015). In neither of these cases were the fitness effects of these behav-

ioral changes documented, e.g., if the changes in males' calls influence their attractiveness to females. Here we determine if female túngara frogs are able to discriminate between infected and uninfected males by attending to the frog's acoustic signal. We did this by recording mating calls of the same males in their infected and uninfected states. This approach is superior to comparing the call of frogs that are either infected or uninfected as it controls for other variables of the males' genotype and phenotype that affect the production of sound. We hypothesized that infection by *B. dendrobatidis* should influence components of the males' sexual mating signal.

3.3 Material and Methods

3.3.1 *B. dendrobatidis* sampling

To test for infection with *B. dendrobatidis* we swabbed the frog's ventral area with a dry swab (Medical Wire and Equipment, model MW110) following established protocols (Hyatt et al., 2007). Swabs were analyzed by qPCR following Boyle et al. (Boyle et al., 2004) and modified by Kriger et al. (Kriger et al., 2006). The swabs were stored in ethanol 70% and analyzed by real-time quantitative PCR (qPCR) using TaqMan® Fast Advanced Master Mix (Applied Biosystems) and a StepOnePlus™ system, with a dilution series of genomic DNA from strain JEL423 as our standard reference.

3.3.2 Male recordings

We collected males in Gamboa in June 2014 and transported them to the University of Texas at Austin, USA. To test for infection with *B. dendrobatidis*, we swabbed each male prior to recording their mating call. We recorded the males in a sound attenuation chamber, placed in a small container with mesh on the sides to allow sound propagation, and with a bowl of water from where the males call. The microphone (Sennheiser ME66) was at 50 cm from the bowl of water. We recorded the males' call with a Zoom H6 recorder, at a sample rate of 44.1 kHz and 16 bit per sample. To record the amplitude of the call, at the beginning of each file we recorded a tone of 780 Hz, measured at an amplitude of 82 dB SPL (re. 20 μPa) at the same position of the bowl of water (50 cm) from where the males call. To stimulate the males to call, we broadcast bouts of a chorus of túngara males recorded in the wild, with silence intervals to be able to record the males' call.

The three males used in the experiments were infected when collected, thus they were also infected prior to the first recording. To clear the males of *B. dendrobatidis*, we treated them with a solution of 0.0025% Itraconazole once a day for 5 min, for 6 consecutive days (Brannelly, 2014). After each 5 min treatment we placed them in a clean container to avoid reinfection. Males were swabbed before the second recording to confirm they were cleared of the infection. We recorded them using the same procedure described above.

To capture the maximum amplitude at which the males were able to call, we chose four calls with the highest amplitude of each recording to mea-

sure their spectral and temporal properties. From each male we chose calls of both recordings, infected and uninfected with *B. dendrobatidis*, with the same structure (whine vs whine, or whine-chuck vs whine-chuck). We measured the amplitude of the call, and temporal and spectral properties as represented in Figure 3.1. For sound analysis we used Raven Pro 1.4 (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY). We performed a principal component analysis (PCA) in R (R Core Team, 2015) to visualize differences in the call parameters depending on the *B. dendrobatidis* infection status of the males.

3.3.3 Female phonotaxis tests

We performed phonotaxis tests in Gamboa, Panamá at the Smithsonian Tropical Research Institute (STRI) from August to December of 2015, using standardized two-choice phonotaxis tests (Ryan & Rand, 1990). We collected pairs in amplexus and we tested the females that same night. We placed females in the center of the sound attenuation chamber (dimensions: 2.7 $\times$ 1.8 m), equidistant from a pair of speakers positioned at 180 ^{o} at opposite ends of the chamber. We video-recorded the tests using an infrared camera placed on the ceiling of the sound chamber.

For the phonotaxis tests we used the same four calls per recording that we analyzed and described above. The three males produced a call with the same structure before and after the treatment (whine/whine or whine-

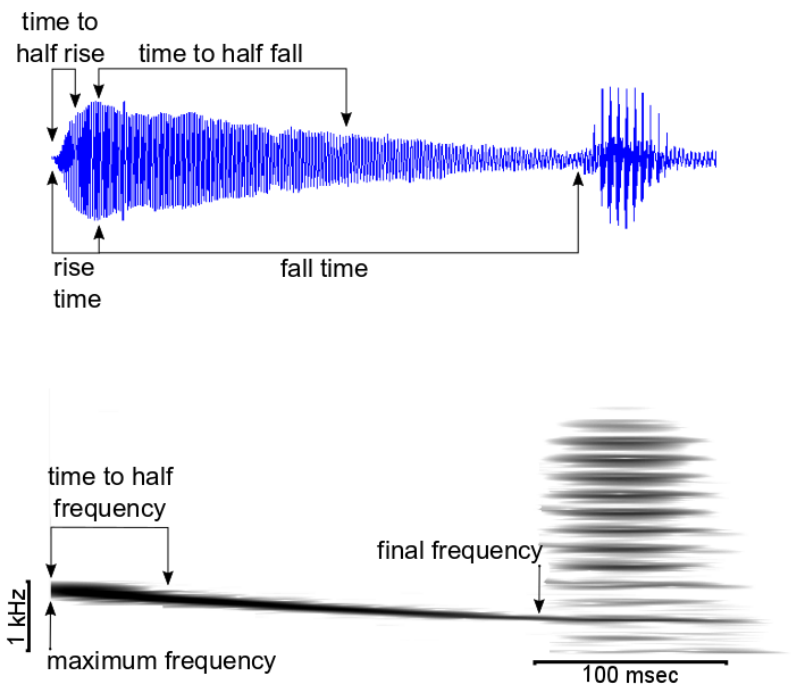

Figure 3.1: Túngara frog males' mating call. Sonogram and spectrogram of a whine and a chuck, and the call variables measured for this study.

chuck/whine-chuck) which we will refer to as males A, B, and C. For the phonotaxis tests playback stimuli, we looped these four calls per recording. For an accurate playback of the relative amplitude at which the males called during the recording, we calibrated each speaker using the tone recorded at the beginning of each file. The tone of each file was played back and measured at 82 dB SPL at the center of the acoustic chamber.

The pair of stimuli was broadcast at a rate of one call per 2 s, alternating from each speaker. We allowed the female to acclimatize for 2 min, under an

inverted mesh funnel. The female was then released, and her preferred stimulus was recorded. We scored the female's preference when the female advanced to within 10 cm in front of one of the speakers. Testers were blind to the treatments broadcast from each speaker.

We tested three versions of the call of the same three males, 'natural', 'normalized', and 'inverted' amplitude calls. The natural calls were played back at the original amplitude at which they were recorded. The normalized and inverted calls, were modified using Adobe Audition 3 (Copyright ©2007 Adobe Systems Incorporated). For the normalized calls, we modified the pairs of calls for each male so that they would have the same peak amplitude. For the inverted calls, we inverted the difference of peak amplitude of the original natural calls of each male (Fig. 3.1). The order of the males' calls was randomized every day. In August, 2015, we tested 66 females with the natural calls. All females completed all three tests, corresponding with the pair of calls from the three males. From October to December 2015 we tested a separate set of 87 females with the normalized and inverted calls. Therefore each female in the second completed six phonotaxis tests.

3.3.4 Statistical Analysis

To determine whether the *B. dendrobatidis* status of males had an effect on female preference for their calls we used a general linear mixed effects models (glmer) with a binomial family and logit link function in R (R Core Team, 2015) using the lme4 package (Bates et al., 2015). Models included male and

female identity as a random factor, and the size (snout-vent length), mass, and *B. dendrobatidis* status of females as fixed factors. We expected the variation in males' mating call due to infection with *B. dendrobatidis* to have a small effect overall on mate choice preference in females. To detect a strength of preference of 0.7 with a statistical power of 0.8 we required a sample size of 42, and of 56 individuals to increase the statistical power to 0.9. Female phonotaxis tests are relatively non-invasive, and the high abundance of túngara frogs in our field sites allow us to have sample sizes (naturals calls, n=66; normalized and inverted calls, n=87) to obtain results with a high statistical power.

3.4 Results

3.4.1 *B. dendrobatidis* infection effects on males' acoustic signals

To test if mating call characteristics varied as a function of infection status, we collected males in the field, in Gamboa, Panamá. We transported the males to the lab to record their mating call inside a sound attenuated chamber. Here we controlled for environmental conditions that could affect the quality of the recording, and we were able to accurately record the amplitude at which the male was calling. High amplitude calls are attractive to females, however they are more energetically costly to produce (Ryan, 1988), thus we expect males in poor condition to produce less attractive calls than healthier males in better condition. We predicted that infection with *B. dendrobatidis* would affect the males' energetic state and therefore influence the amplitude of the males' mating call. Prior to recording, we swabbed each male to test for *B. dendrobatidis* infection, following a standard swabbing protocol (Hyatt et al., 2007) and analysis using real-time quantitative PCR (qPCR) (Boyle et al., 2004; Kriger et al., 2006). We recorded the males' calls for at least one hour. From each of these recordings we chose the four calls with the highest amplitude, and measured their spectral and temporal properties as shown in Figure 3.1.

Table 3.1: Average values for measured properties of the mating calls of three túngara males when infected with *B. dendrobatidis* [*Bd* (+)] vs when uninfected [*Bd* (-)].

Male	*Bd*	Time to half frequency (s)	Frequency Dominant (Hz)	Frequency Initial (Hz)	Frequency Final (Hz)	
A	+	0.137	861.3	882.8	430.7	
A	-	0.069	753.6	818.2	430.7	
B	+	0.064	947.5	1098.2	516.8	
B	-	0.048	775.2	861.3	473.7	
C	+	0.078	861.3	947.5	516.8	
C	-	0.050	818.2	861.3	559.8	
Male	*Bd*	Time to half fall (s)	Time to half rise (s)	Rise time (s)	Fall time (s)	Amplitude
A	+	0.042	0.008	0.025	0.272	813.20
A	-	0.100	0.006	0.021	0.341	1774.10
B	+	0.028	0.005	0.014	0.281	838.38
B	-	0.028	0.005	0.014	0.281	938.25
C	+	0.128	0.003	0.038	0.248	873.43
C	-	0.193	0.004	0.011	0.292	1806.20

All males that we recorded were infected with *B. dendrobatidis*. After obtaining the recording of their mating call we treated them for six days with an antifungal, Itraconazole, to clear the infection (Brannelly, 2014). The success of the treatment in clearing the infection was confirmed by qPCR (Boyle et al., 2004; Kriger et al., 2006). Once the males were cleared of the infection, we recorded their mating calls again using the same procedure as described above. In this way we were able to control for variation in the call parameters due to aspects of the call related with the males' phenotype.

We compared the call parameters of the same male in its infected and not-infected state. For this analysis we compared only calls with the same structure, either whine vs whine, or whine-chuck vs whine-chuck. Three males produced a call with the same structure for both infection states. The parameters we measured are represented in Figure 3.1: the time to half rise (duration from the onset of the call to one-half the maximum amplitude during the rise), the time to half fall (time from the beginning of the call to the point at one-half the maximum amplitude during the fall), rise time (time from the whine's onset to its maximum amplitude), fall time (time from the whine's maximum amplitude to the end of the whine), time to half frequency (whine's duration from the onset to its mid-frequency), final frequency (whine's frequency at the end of the whine), maximum frequency (maximum frequency of the whine's fundamental), dominant frequency (dominant frequency of the call), and amplitude (root-mean-square amplitude).

The mating calls of males when infected with *B. dendrobatidis* had a lower amplitude and higher dominant frequency than the calls of the same males when not infected (Table 3.1). We used principal components analysis to summarise the multivariate properties of the six calls. The first principal component explained 38% of the variation amongst the calls (Fig. 3.2; Table 3.2). Along this axis, calls grouped by infection status rather than individual. In addition, amplitude and dominant frequency of the whine had the higher loadings on this axis. We then asked if these differences in the characteristics of the call affect the males' call attractiveness to females.

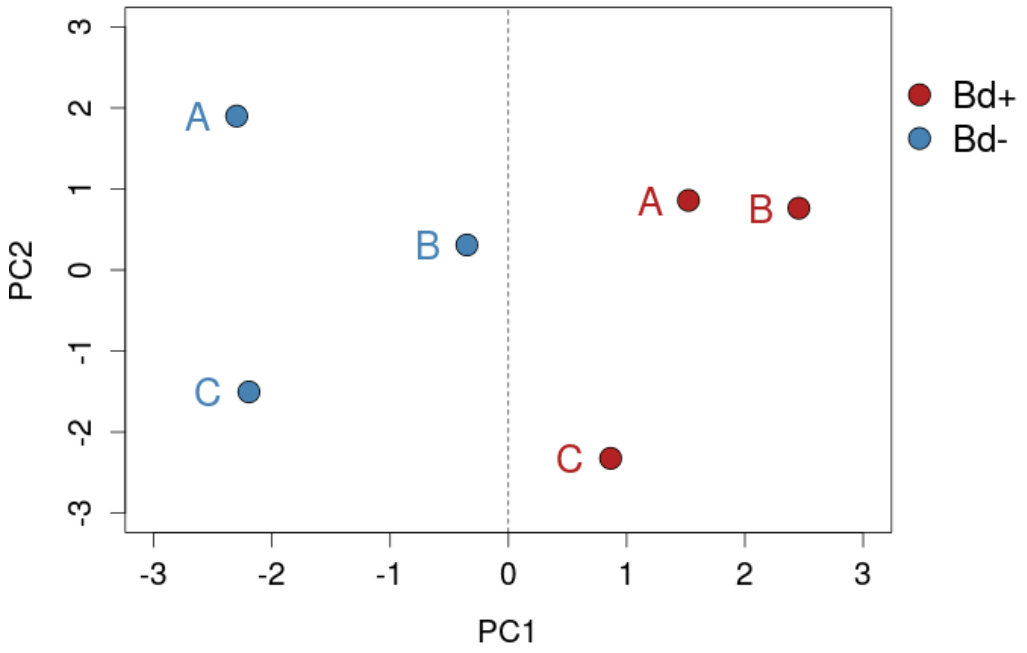

Figure 3.2: Principal components analysis of the multivariate properties of mating calls of túngara frogs. Call properties of mating calls of three males: A, B and C, in their infected with *B. dendrobatidis* (red) vs uninfected with *B. dendrobatidis* (blue) status. First principal component (PC1) had high loadings for amplitude of the call and the whine's dominant frequency.

Table 3.2: Principal component analysis summary of the multivariate properties of the mating calls of túngara males measured in this study. Amplitude and dominant frequency of the whine have the highest loading in the first principal component.

	PC1	PC2	PC3	PC4	PC5
Standard deviation	0.966	1.594	1.471	0.934	0.749
Proportion of variance	0.386	0.254	0.216	0.087	0.056
Cumulative proportion	0.386	0.640	0.857	0.944	1.000
Loading values					
Time to half frequency	0.234	0.134	-0.444	0.349	0.611
Dominant frequency	**0.432**	-0.113	0.267	0.251	0.262
Initial frequency	0.405	-0.065	0.383	0.157	-0.168
Final frequency	-0.013	-0.477	0.436	-0.060	0.123
Time to half fall	-0.313	-0.394	0.119	0.418	0.275
Time to half rise	0.296	0.454	0.210	-0.070	0.264
Rise time	0.205	-0.209	-0.388	0.545	-0.503
Fall time	-0.386	0.389	0.050	0.196	-0.043
Amplitude	**-0.466**	0.041	0.171	0.279	0.209

3.4.2 Females discriminate against *B. dendrobatidis*-infected males

To determine if females can discriminate against the calls of males when males are infected with *B. dendrobatidis*, we tested females in a standard two-choice phonotaxis test. Gravid females were collected in the field and tested the same night. In the first experiment 66 females responded to three tests, one for each pair of calls from the three males, for a total of 198 phonotaxis tests. Each pair consisted of the mating call produced by a male while infected with *B. dendrobatidis* versus the call of that same male after he was cleared of the infection with *B. dendrobatidis*. As these calls were broadcast at the same amplitude

as they were recorded, we refer to them as 'natural calls'. In this experiment, females discriminated between *B. dendrobatidis*-infected and uninfected males (Fig. 3.3; Table 3.3). This is not surprising since *B. dendrobatidis*-infected males called at lower amplitudes (Table 3.1) and female frogs often are attracted to call with higher amplitudes (Gerhardt & Huber, 2002).

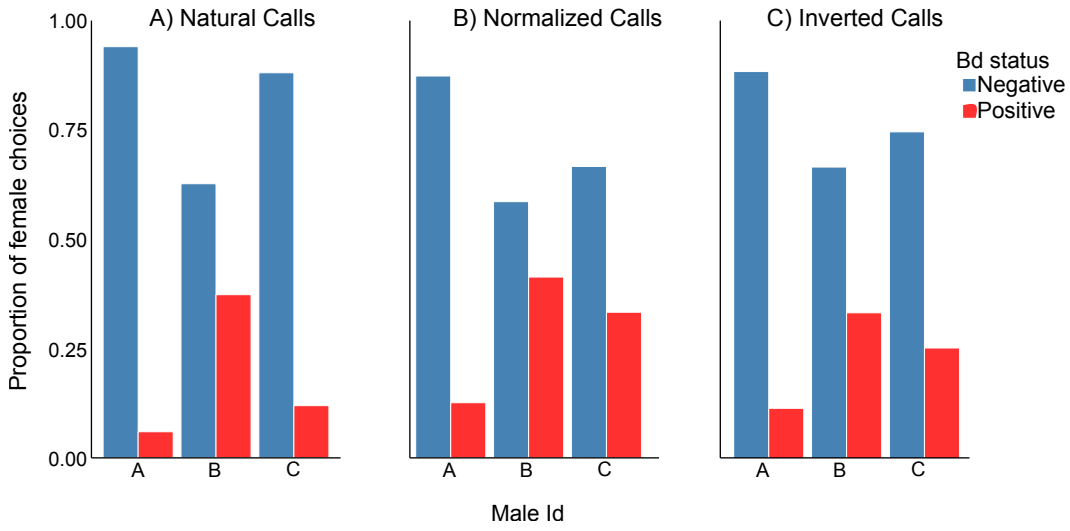

Figure 3.3: Female preference for calls of males uninfected and infected with the chytrid pathogen *B. dendrobatidis* in phonotaxis tests. Female túngara frogs prefer the calls of uninfected males (blue bars) vs the calls of the same males when infected with *B. dendrobatidis* (red bars). Proportion of female choice is shown for three types of calls: A) natural calls (n=66), B) amplitude normalized calls (n=87) and amplitude inverted calls (n=87).

To determine if call amplitude was the salient feature influencing female mate choice, in the second and third experiments we manipulated the amplitude of the calls independent of other spectral or temporal characteristics

of the acoustic signal. For the second experiment we broadcast both calls of a pair at the same peak amplitude, and for the third experiment we inverted their peak amplitude. We will refer to this as 'normalized amplitude calls' and 'inverted amplitude calls', respectively. A different group of females, a total of 87, were tested in these two experiments, and responded to all of the 6 tests for a total of 522 phonotaxis tests. In both sets of experiments females showed a preference for the calls of males after the infection was cleared. When the calls were normalized, the females still preferred the calls of the males in their uninfected state (Fig. 3.3). Similarly, when the call amplitudes were reversed, such that the call produced while the male was infected with *B. dendrobatidis* was louder, the females still preferred the call produced after the infection was cleared (Fig. 3.3). Consistent with the results of the tests with the natural calls, these preferences remained the same for each male: females preferred the call of the male when it was clear of the infection over the call of the same male when infected (Table 3.3). These results show that, in addition to the amplitude of the mating call, other components of the call contribute to female mating preference of uninfected males.

Table 3.3: Effect of *B. dendrobatidis* infection status of males on females' preference. Summary of the general linear mixed-effects model to test for the effect of *B. dendrobatidis* infection status of males on female preference for the three experiments: natural calls, inverted amplitude, and normalized amplitude.

	Estimate	Standard Error	z value	p
Natural calls				
Intercept	-1.605	0.569	-2.819	**0.005**
Female size	-0.095	0.235	-0.402	0.688
Female weight	-0.426	0.721	-0.590	0.555
Female *Bd* status	-0.555	0.575	-0.965	0.335
Inverted amplitude				
Intercept	-1.477	0.430	-3.433	**0.001**
Female size	0.050	0.086	0.582	0.560
Female weight	-0.421	0.457	-0.921	0.357
Female *Bd* status	-0.460	0.511	-0.899	0.368
Normalized amplitude				
Intercept	-1.177	0.450	-2.617	**0.009**
Female size	-0.139	0.087	-1.601	0.109
Female weight	0.021	0.398	0.053	0.958
Female *Bd* status	0.064	0.410	0.156	0.876

3.5 Discussion

Here we demonstrate that the effect on the properties of the males' mating call of túngara frogs caused by the infection with *B. dendrobatidis* is salient to females and they discriminate against these calls. The female preference for calls of uninfected males suggests that there is an associated cost of infection with *B. dendrobatidis* in the male's performance, in this case producing a mating

call. Thus infection with *B. dendrobatidis* incurs a cost in the attractiveness of mating calls, and might reduce their fitness. Since *B. dendrobatidis* infects amphibians skin, mating with an infected male increases the probability of infection. Females that discriminate against *B. dendrobatidis*-infected males may decrease their chance of transmission of *B. dendrobatidis*. Other studies have suggested that infection with *B. dendrobatidis* changes calling behavior, where calls of males infected with *B. dendrobatidis* might be more attractive, but the ultimate effect on female preference was not tested. In the Japanese tree frog, *Hyla japonica,* males infected with *B. dendrobatidis* produced more pulses per note and longer note duration suggesting a higher calling effort when infected (An & Waldman, 2016). The Australian frog *Litoria rheocola*, is more likely to be found calling if males are infected and in good condition (Roznik et al., 2015), although the properties of the mating call did not differ between infected and uninfected males (Greenspan et al., 2016). Although both studies suggest that these changes in calling behavior due to infection with *B. dendrobatidis* may increase the males' attractiveness, and therefore increase the dispersion of *B. dendrobatidis*, female preference tests were not performed so these assertions remain to be tested. Our study shows evidence that sub-lethal effects of infection with *B. dendrobatidis* on apparently resistant species should decrease males' Darwinian fitness by making them less attractive to females, and may aid females in avoiding other potential sub-lethal effects of chytridiomycosis.

3.6 Acknowledgments

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Chapter 4

Condition-dependent effects of chytridiomycosis in the reproductive output of male túngara frogs

4.1 Abstract

Amphibians worldwide are experiencing massive population declines and extinctions. One of the main causes is chytridiomycosis, a disease caused by the chytrid fungus *Batrachochytrium dendrobatidis.* Most research and conservation efforts are focused on the lethal aspects of the disease in critically vulnerable species. However, there is growing evidence that chytridiomycosis might have sub-lethal effects in less susceptible or resistant species with potentially long-term population-level consequences. In this study we assessed whether the infection with *B. dendrobatidis* in adults affects their reproductive output. Based on recent evidence that infection with *B. dendrobatidis* decreases the attractiveness of males' mating calls, we hypothesized that pathogen-induced stress might incur a trade-off in investment between the immune response and reproduction. The presence of such trade-off might affect the body condition of males and females, which in turn might have consequences on their reproductive output. We found that males that are infected but in good body condition had a higher number of offspring, but their tadpoles were on average smaller. Thus, it appears that in male túngara frogs there is a condition-dependent cost

of the infection with *B. dendrobatidis,* probably mediated by the allocation of resources into the immune response. However, the effect that body condition of infected males has on their offspring, specifically the trade-off between number and size of tadpoles, needs to be further studied.

4.2 Introduction

Chytridiomycosis is a disease believed to be responsible for the worst vertebrate mass-mortalities and extinctions in human history (Ceballos et al., 2015; Kolbert, 2014; Voyles et al., 2009; Wake & Vredenburg, 2008). It is caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Longcore et al., 1999), whose infective life stage is a waterborne flagellated zoospore. This fungal pathogen infects amphibians upon colonization of keratinized tissue; that is, the mouthparts of larvae and the skin of adults. Chytridiomycosis causes osmoregulatory irregularities on the skin of adults (Voyles et al., 2009), which seems to be the main cause of death, but it also affects their immune system (Fites et al., 2013). The effects of chytridiomycosis, as in the case of most diseases, are context-dependent on the interaction of host, pathogen, and the environment. Endogenous factors like the identity of the host and the strain of the pathogen affect the virulence of the pathogen and the susceptibility of the host. Moreover, the complex life cycle of amphibians with a wide variety of life histories adds to the complexity of this interaction (Stockwell et al., 2010).

Chytridiomycosis is well known for its lethal effects, but some studies have shown non-lethal responses to the infection during the larval and adult stage, with possible long-term effects on the life history of anurans (Garner et al., 2009). As individuals metamorphose from tadpoles to froglets, there are major morphological and physiological changes. Some of these changes are the keratinization of the skin and the reorganization of the immune system. Infection with *B. dendrobatidis* was observed to elevate the concentration of corticosterone in infected tadpoles of two species of *Alytes* (Gabor et al., 2013), and in adults of *Litoria caerulea* (Peterson et al., 2013). The allocation of energy and resources into immunocompetence could cause a trade-off with other aspects of life history (McCallum, 2012; Norris, 2000). Thus, frogs infected with the pathogen might allocate resources into fighting the infection at the expense of allocating less resources into reproduction. While it is clearly important to study the morbidity and mortality effects of a pathogen, especially one implicated in global mass declines and extinctions, it is also important to understand its non-lethal effects that although less severe might nonetheless have lasting population-level consequences.

The well-characterized natural history of the túngara frog presents an interesting opportunity to expand our understanding of the non-lethal effects of the infection with *B. dendrobatidis*. Túngara frog (*Physalaemus pustulosus*) populations in Panama are infected with *B. dendrobatidis* (Rodríguez-Brenes et al., 2016), but symptoms of chytridiomycosis and population declines have not been observed. The eggs of túngara frogs develop in a foam nest that

protects them against dehydration and direct exposure to light (Ryan, 1985). During mating, the pair in amplexus floats on the water as the female releases eggs. The male releases sperm but also invests energy by beating the egg mass with his hind legs to produce the foam.

Elsewhere, we have shown in túngara frogs that *B. dendrobatidis* infection can reduce the attractiveness of the male's mating call (Rodríguez-Brenes et al. *in review*). The decrease in attractiveness, a non-lethal effect, could be due to allocation of energy towards the immune response against the pathogen, and therefore lowering of the investment in reproduction aspects like the production of acoustic signals (Martin et al., 2003; Zuk & Stoehr, 2002). The goal of this study was to examine the potential for other sub-lethal effects that infection of *B. dendrobatidis* might have on reproductive behavior of túngara frogs. It is possible that the infection status of mated males or females has a direct effect on their reproductive output. We hypothesize that infection with *B. dentrobatidis* causes pathogen-mediated stress resulting in a trade-off between immune response and reproduction. To evaluate this hypothesis, we studied the infection status and body condition of adults and correlated these to the reproductive output of adults (number of offspring) as well as the body length of their tadpoles during the first weeks of development.

4.3 Methods

We collected pairs of túngara frogs in amplexus in Gamboa, Panama from October to November of 2016. To test if they were infected with *B. den-*

drobatidis, we swabbed the male's and female's ventral skin with a dry swab (Medical Wire and Equipment, model MW110) following established protocols (Hyatt et al., 2007). Swabs were analyzed in triplicate for presence of *B. dendrobatidis* by quantitative polymerase chain reaction (qPCR) following Boyle et al. (2004) and modified by Kriger et al. (2006). Females were used in phonotaxis tests (Rodríguez-Brenes et al. *in review*) the same night that they were collected and swabbed. After the phonotaxis test, we measured the body mass (g) and snout-vent length (SVL, mm) of males and females. The pairs were thereafter kept in a plastic container with a small amount of clean water and allowed to complete their mating and build a foam nest. Foam nests were transferred to a plastic container with clean water and the adult frogs were released at the same site where they were collected.

Tadpoles hatched within three days. For each foam nest we recorded the total number of tadpoles that hatched, and after approximately seven days we collected three tadpoles per clutch and preserved them in ethanol 70%. We took pictures of the tadpoles with a stereoscope and measured their body length (BL) using ImageJ (Schindelin et al., 2015). As a proxy of the overall quality and energetic state of the adults, we used a body condition index of males and females. For females we also estimated performance as the latency to choice during phonotaxis tests. We calculated the body condition index of adults as the residuals of the linear regression of the cube root of body mass on SVL, and then dividing the residuals by the SVL of each individual (Bernal et al., 2007). For the latency of females during phonotaxis tests, we calculated

the average time that each female took to choose between two acoustic stimuli in replicate phonotaxis tests (Rodríguez-Brenes et al., *in review*).

We tested whether the infection with *B. dendrobatidis* affected: the body condition index of males and females, the number of tadpoles per foam nest, and the tadpoles' average body length using Kruskal-Wallis tests. We also tested if the number of tadpoles per foam nest and the tadpoles' body length was related to (i) the parents' body condition index or (ii) the females' latency using linear regressions. Because the infection status of adult frogs and tadpoles was unknown until the qPCR was performed, all experiments were blind with respect to the hypothesis to be tested. All statistical analyses were done using R (R Core Team, 2015).

4.4 Results

We obtained foam nests from 29 pairs. In seven of them only one of the parents was infected with *B. dendrobatidis* (female only = 1, male only = 6). In five pairs both adults were infected, and two of their foam nests had infected tadpoles. In 17 pairs neither adult was infected, but four of the foam nests produced by these pairs had a single infected tadpole. The body condition index of males and females was not associated with their infection status (H=5.441, p=0.142, n=29, Fig. 4.1. The infection status of either the female or male in the pair did not appear to affect the number of tadpoles that hatched per foam nest (*B. dendrobatidis* status: H= 2.011, p=0.570, n=29, Fig. 4.2), or the tadpoles' body length (H= 1.704, p=0.628, n=29, Fig. 4.2).

There was no relation between the number of tadpoles that hatched per foam nest (Fig. 4.3) or their body length (Fig. 4.4), with the body condition of infected or non-infected females. The number of tadpoles that hatched per foam nest was positively correlated with the body condition index of infected males, but was there was no relationship between the body condition index of uninfected males and their reproductive output (Fig. 4.3). The body length of the tadpoles was positively correlated with the male's body condition index when males were not infected (Fig. 4.4). The opposite was observed in infected males; there was a negative correlation between the body condition index and the number of tadpoles that hatched per foam nest (Fig. 4.4). Neither the number of tadpoles, nor their body length was correlated with females' latency during phonotaxis tests (Fig. 4.5).

Table 4.1: Summary of linear regression for number of tadpoles per foam nest against the body condition index of the males and females grouped by their infection status with *Batrachochytrium dendrobatidis* (Bd).

Sex	Bd status	R^2	d.f.	P value
Female	Negative	0.0073	2	0.697
	Positive	0.0309	2	0.739
Male	Negative	0.0115	2	0.671
	Positive	0.5571	2	0.008 *

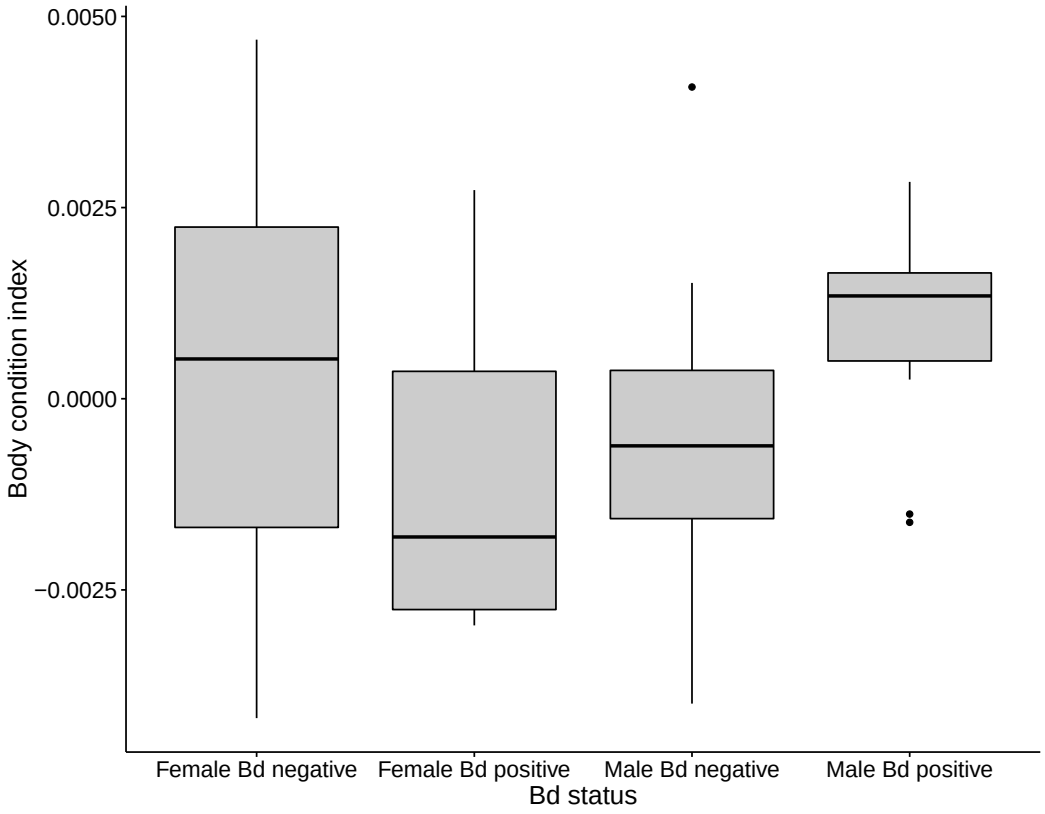

Figure 4.1: Body condition index of male and female túngara frogs that were infected (*B. dendrobatidis* positive) and not-infected (*B. dendrobatidis* negative) (n=29).

Table 4.2: Summary of linear regression for the average body length of tadpoles (mm) against the body condition index of the males and females grouped by their infection status with *B. dendrobatidis* (Bd).

Sex	Bd status	R^2	d.f.	P value
Female	Negative	0.0029	2	0.806
	Positive	0.0009	2	0.955
Male	Negative	0.3834	2	0.006*
	Positive	0.6629	2	0.002*

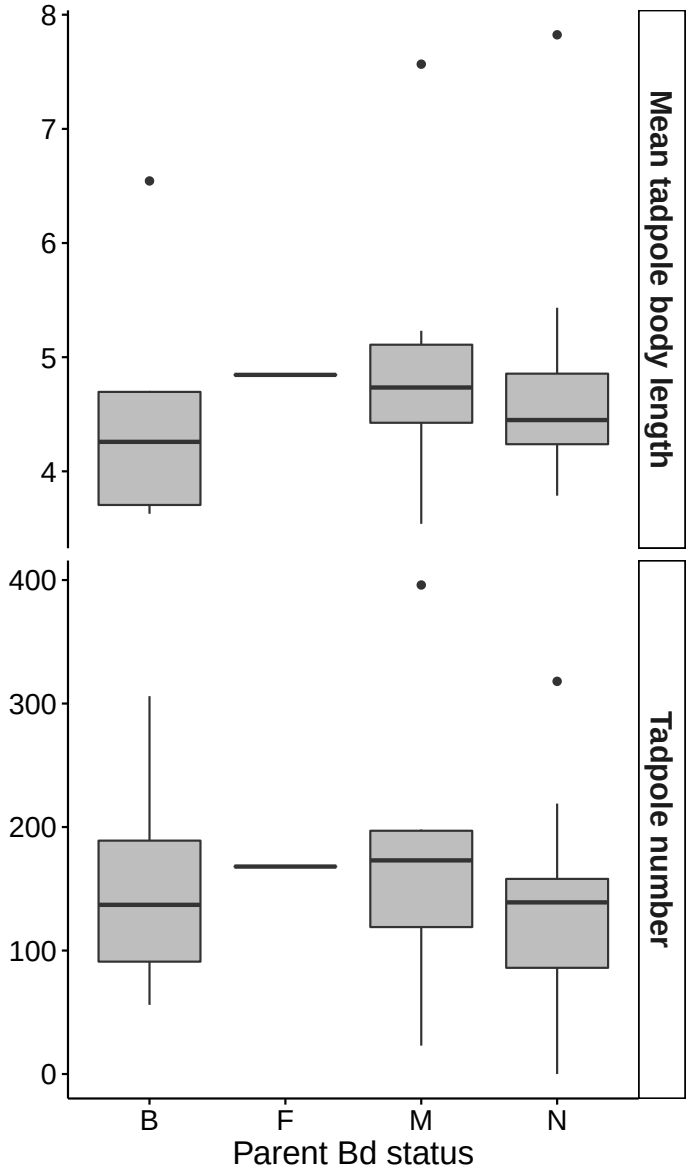

Figure 4.2: Effect of the parents' *B. dendrobatidis* infection status [both infected (B, n=5), female (F, n=1) or male (M, n=6) only, or none of them infected (N, n=17)] on the number of tadpoles that hatched per foam nests (bottom), or the tadpoles body length (mm) (top).

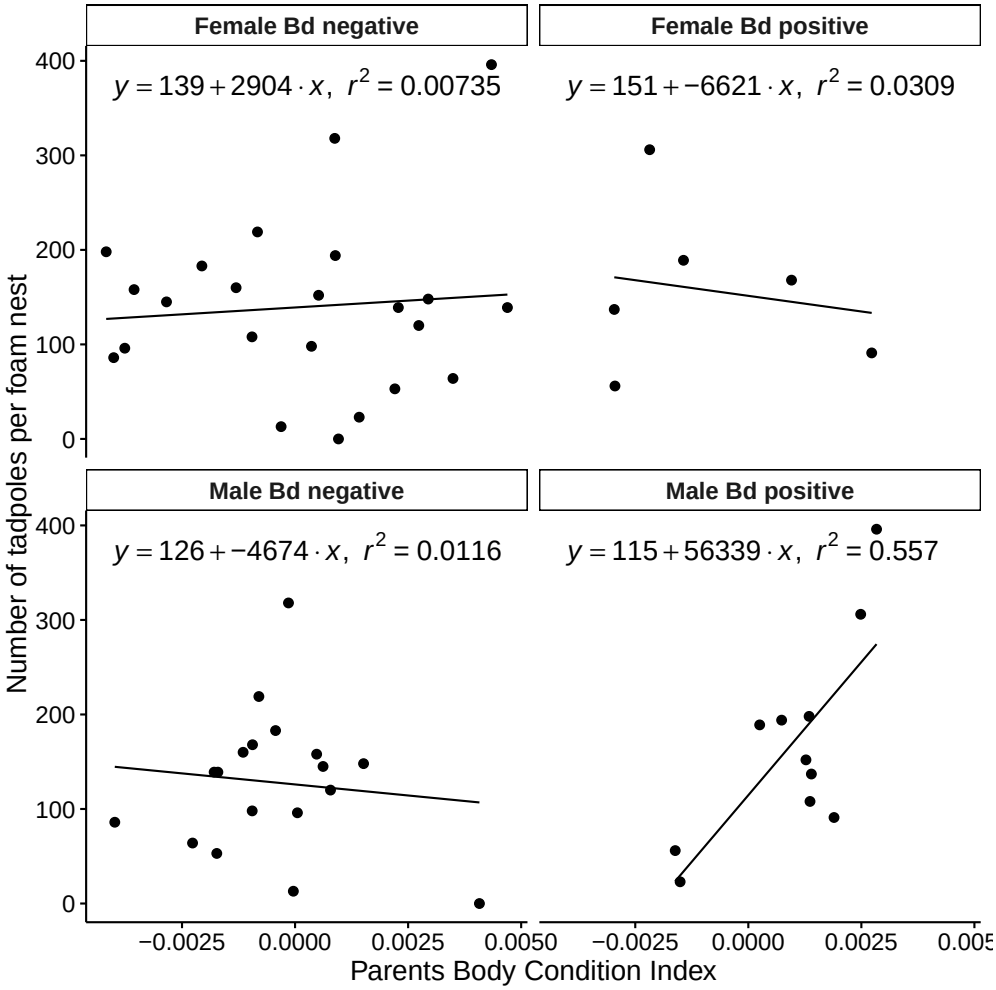

Figure 4.3: Number of tadpoles per foam nest as a function of the body condition of the males and females and grouped by their infection status with *B. dendrobatidis* (Bd).

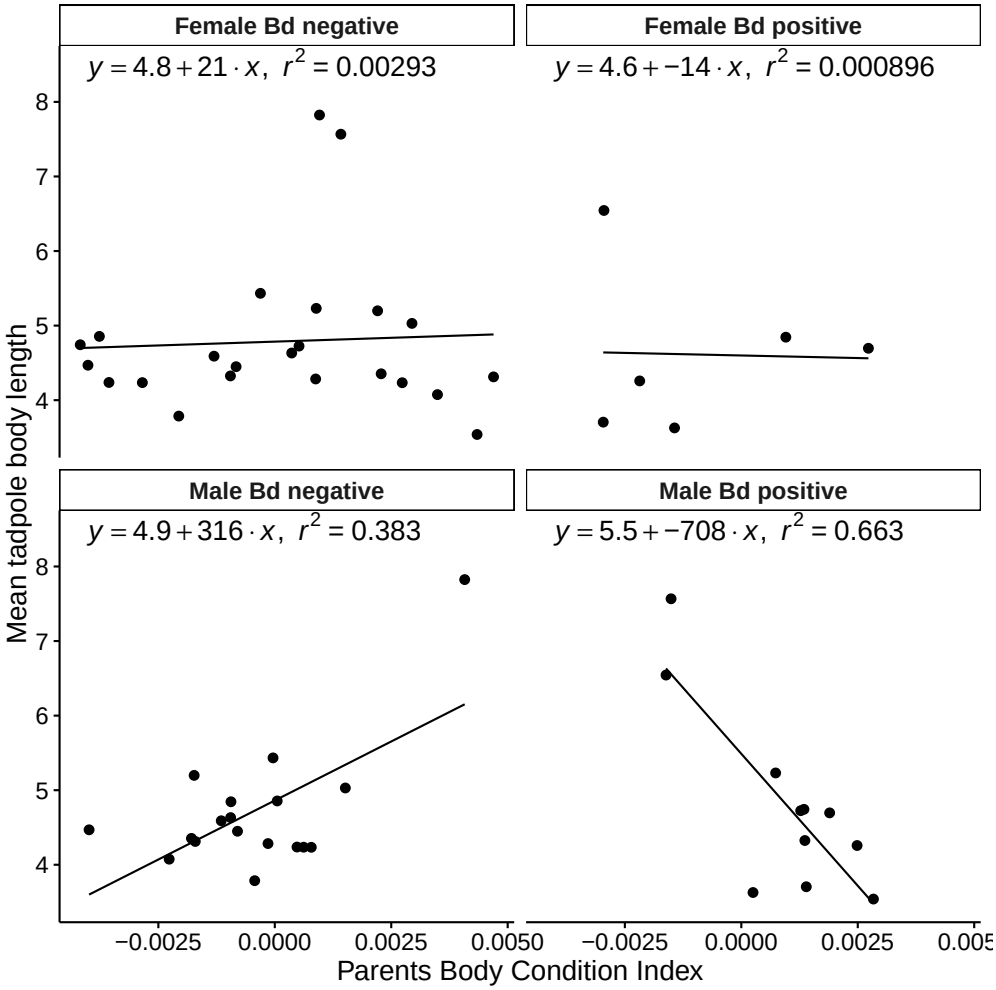

Figure 4.4: Average body length of tadpoles (mm) as a function of the body condition index of the males and females grouped by their infection status with *B. dendrobatidis* (Bd).

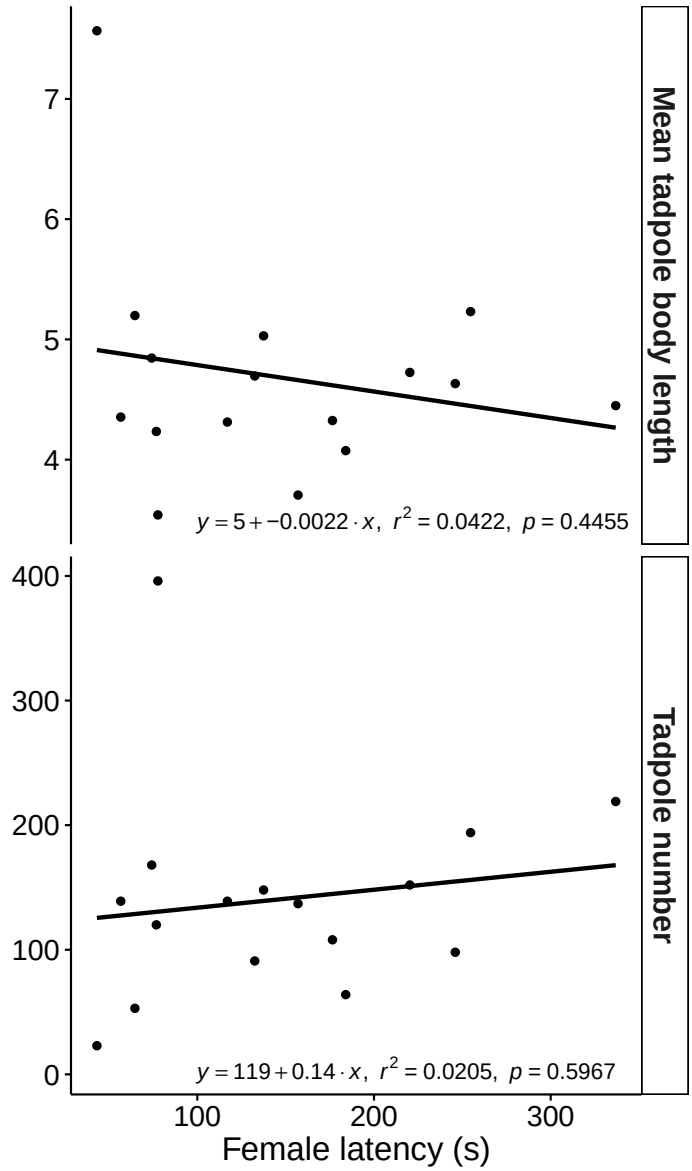

Figure 4.5: Female latency, time to make a choice in phonotaxis tests (n=16), as a predictor of tadpole number and tadpole body length (mm).

4.5 Discussion

To test for the effect that parental infection with *B. dendrobatidis* could have on offspring, we used (i) the adults' infection status, (ii) adults' body condition index, and as a measure of performance, (iii) females' latency during phonotaxis tests. These three variables were tested as potential predictors for (i) the number of tadpoles that hatched per foam nest and (ii) the tadpoles' body length. The infection status of the parents did not predict their body condition (Fig. 4.1), the number of tadpoles, or their tadpoles' body length (Fig. 4.2). However, the effect of parents' body condition on the offspring differed between the two sexes and depended on their infection status. Non-infected males in good body condition had larger tadpoles than males in poorer body condition (Fig. 4.4), but the number of tadpoles was not affected (Fig. 4.3). These results suggest that a male's body condition affects its investment in offspring. Moreover, in infected males, we observed that the number of tadpoles that hatched per foam nest increased as the males' body condition increased (Fig. 4.3), but the body length of the tadpoles decreased as the body condition of males increased (Fig. 4.4). These results suggest that for infected males there exists a trade-off between the number of tadpoles they can produce and the body length of those tadpoles.

When in good condition, infected males can invest sufficiently to produce a high number of tadpoles, but this comes at the cost of lower body length of those tadpoles. However, the proximal causes of this phenomenon are unclear. Male túngara frogs invest energy in building the nest, but the ef-

fect of the quality of the foam nest on tadpoles has not been studied. In other species of frogs where the tadpoles develop in foam nests, there is evidence that tadpoles benefit from the foam nest. In *Rhacophorus viridis viridis*, starved tadpoles have been documented to ingest either the foam nest, or microorganisms attached to the foam nests (Tanaka & Nishihira, 1987). Similarly, in *R. arboreus*, tadpoles that only had foam and were not fed grew as much as tadpoles that were been fed regularly (Kusano et al., 2006). We have no evidence that this could be the case for tadpoles of *P. pustulosus,* as there are no studies that look at the role of the foam nests as a source of nutrition. Further studies are necessary to disentangle the role of the male outside physically constructing the foam nest and providing sperm, and whether the foam nest is only a physical barrier against environmental conditions or if it has some nutritional value.

In general, the growth rate of tadpoles is positively correlated with the size at metamorphosis and adult size, and smaller size at metamorphosis decreases the chances of survival (Altwegg et al., 2003). Even if males produced more tadpoles, if these are smaller at metamorphosis, then the effect of infection in the adult male could be carried on in the tadpoles. In addition, exposure or infection with *B. dendrobatidis* could negatively affect the development of the tadpoles. Smaller body size at time of metamorphosis in the common toad, *Bufo bufo,* as a result of exposure and infection with *B. dendrobatidis*, increased mortality in the tadpoles (Garner et al., 2009). As we only collected pairs in amplexus, all the males that we tested had suc-

cessfully mated with a female, suggesting that they successfully overcame the trade-off between the immune system and reproduction, and were capable of simultaneously fighting off the infection and producing attractive mating calls (Sheldon & Verhulst, 1996). In Figure 1, we can observe that among the males that successfully mated, infected males seem to be in better condition than non-infected males. Similar to our results for túngara males, in the treefrog *Litoria rheocola* the calling effort of males infected with *B. dendrobatidis* was explained by their body condition index. Infected males in good body condition were more likely to call than infected males in poor body condition (Roznik et al., 2015). The trade-off between the immune response to diseases and parasites and the allocation of energy and resources into other aspects of life history has been tested in various taxa. The call rate of male tree frogs of the species *Hybsiboas prasinus* decreased in individuals with higher loads of helminth parasites (Madelaire et al., 2013). An induced immune activity was sufficient to reduce the parental effort of females of the pied flycatcher, *Ficedula hypoleuca* (Ilmonen et al., 2000), and to increase the metabolic rate of the white cabbage butterfly pupae, *Pieris brassicae* (Freitak et al., 2003), and the house sparrow, *Passer domesticus* (Martin et al., 2003). These results show that there is an associated metabolic cost of the immune response that could directly or indirectly affect the investment into reproductive effort.

Calling activity in frogs is energetically costly (Gerhardt & Huber, 2002), and the role of male túngara frogs in building the foam nest, by beating the eggs mass to form the foam, is also energetically demanding (Ryan,

1985). Our results on male túngara frogs reproductive output and those on *L. rheocola* calling effort reinforce the idea that males require good body condition to mount an immune response against *B. dendrobatidis*, and at the same time allocate energy and resources into aspects of reproduction like mate attraction, reproductive output, and thus fitness. Our results show that there is non-lethal cost of the infection with *B. dendrobatidis*, a pathogen better known for its lethal effects on a vast number of amphibian species. In the long term, these non-lethal costs may take part in the evolution of the immune responses of amphibian species to *B. dendrobatidis*.

Chapter 5

Conclusion

Chytridiomycosis is an infectious disease believed to be responsible for the greatest vertebrate mass-mortality and extinction in human history. According to the Global Amphibian Assessment (Stuart et al., 2008), approximately one third (32%) of frogs in the world are threatened, 43% of the amphibian populations are declining, 168 species of amphibians are believed to be extinct, and the numbers are rising. Most research on chytridiomycosis deals with the lethal effects of the disease: the epizootic and enzootic dynamic across sites where the effects of *B. dendrobatidis* have been catastrophic. In this dissertation I document the geographic distribution of *B. dendrobatidis* in populations of the túngara frog in lowland areas of Panama. I expected that, as with other host-pathogen systems, infections with *B. dendrobatidis* in túngara frogs, might have consequences on the host's populations. Therefore, I also investigated the effects of the infection with *B. dendrobatidis* on important aspects of the reproductive behavior and fitness of túngara frogs. The in-depth understanding of the reproductive behavior of the túngara frogs allowed me to measure the effects of *B. dendrobatidis* on its reproductive biology.

In the lowlands of central and eastern Panamá, the pathogen spread

rapidly. Within four years, *B. dendrobatidis* dispersed from central Panamá (2011) to the remote areas in the Darién National Park, Eastern Panamá (2014), where it hadn't been documented before. The presence of the pathogen in the Darién region closes the gap of its distribution between Central and South America. Although the warmer lowland habitats sampled here offer less favorable conditions for growth of *B. dendrobatidis*, the chytrid spread across the lowlands at rates similar to those reported for the highlands. However, *B. dendrobatidis* virulence is lower in the lowlands where temperature is comparatively higher than in the highlands. During my six-year survey, I screened 1695 frogs for the presence of the pathogen, but I did not detect visible symptoms of chytridiomycosis in túngara frogs from Central and Eastern Panama, nor did I observe population declines that are common in other species.

Species that carry *B. dendrobatidis* asymptomatically and share the habitat with more vulnerable species can potentially function as reservoirs of the pathogen. The túngara frog is one of the most common species throughout Central America, inhabiting disturbed areas like the populations in Chiriquí and Gamboa, and undisturbed areas like the Darién Gap. The wide diversity of areas that túngara frogs inhabit makes them an ideal reservoir and disperser for the pathogen, sharing the habitat with other vulnerable and non-vulnerable frog species. As I show in this study, the non-lethal infection by *B. dendrobatidis* in apparently resistant species can potentially have long term effects as they ultimately affect individuals' fitness. In males of the túngara frog, the

infection causes significant differences in the quality of the mating call, and this effect is sufficient to influence female preferences. Female túngara frogs discriminated against the calls of infected males, suggesting that there exists a cost to males if infected by *B. dendrobatidis*. Such an effect on the properties of the males' call was probably due to an investment trade-off between the immune response to the pathogen and reproductive behavior. Males that cannot balance this trade-off are less attractive to females, and ultimately have lower fitness.

Since *B. dendrobatidis* infects amphibian skin, mating with an infected male increases the probability of infection. Females that discriminate against Bd-infected males may decrease their chance of transmission of *B. dendrobatidis*, but reduce the risk of infecting their offspring. Males that are infected with *B. dendrobatidis* and are in good body condition, had higher reproductive output, but the body length of their tadpoles was smaller. While my results demonstrate that males that can bear the costs of the immune response against *B. dendrobatidis* invest more in reproductive output, more research is necessary to understand how does relative increase in tadpole number interact with the initial body size and growth rate of tadpoles. Overall, these studies document that sub-lethal effects of the infection with *B. dendrobatidis* in tolerant species could decrease males' Darwinian fitness by making them less attractive to females, and may aid females in avoiding other potential sub-lethal effects of chytridiomycosis to their offspring.

In summary, this research i) documents the dynamic of epizootic and

endozootic history of *B. dendrobatidis* in populations of the túngara frog along the lowlands in the tropics, ii) contributes to the understanding of the physiological and behavioral trade-offs confronted by a species during response to a pathogen, iii) determines if *B. dendrobatidis* can have long-term and perhaps subtle effects in apparently resistant species. Characterization of non-lethal infections by *B. dendrobatidis* represents an important contribution towards the understanding of the general effects of chytridiomycosis in amphibians. More broadly, the work presented here contributes to the field of emerging infectious diseases which, in addition to frogs and salamanders, are affecting a number of other ecologically and economically important lineages including honeybees, bats, birds, and humans.

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Vita

Sofía Rodríguez Brenes was born and grew up in San Jose, Costa Rica. She pursued undergraduate studies at the Escuela de Biología, Universidad de Costa Rica (UCR). Early in her career, her interest in ecology and behavior of vertebrates, especially amphibians, developed into a passion for field research and natural history. This was reinforced when Sofía participated in the field course Ecología Tropical y Conservación from the Organization for Tropical Studies (OTS), which strongly shaped her research interests and skills. As an undergraduate student she worked as a teaching and research assistant in Costa Rica and Panamá on several projects including anuran population declines and chytridiomycosis. Sofía is passionate about teaching in the field and after graduating from UCR she worked as a field instructor for a science and conservation program for high school students in Costa Rica and Mexico. She maintains her field teaching activities through OTS Talent Identification Program courses in which she teaches tropical ecology for high school students in Costa Rica. In 2010 Sofía started her PhD studies in Ecology, Evolution and Behavior at the University of Texas at Austin, where she applied her field experience to study the interaction between frogs and the chytrid pathogen.

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